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Susceptibility of *Coxiella burnetii* to Chemical Disinfectants

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INTRODUCTION

The resistance of *Coxiella burnetii* to physical and chemical stress is well documented.[1,2] Compared with vegetative bacteria and other rickettsiae, this hardy microorganism resists elevated temperatures, desiccation, osmotic shock,[3] ultraviolet light, and chemical disinfectants.[4] The environmental stability of *C. burnetii* is perhaps related to (i) the sporulation process, which produces endogenous spores and small dense cells,[5] and (ii) the metabolic dormancy of *C. burnetii* at neutral pH.[6] *C. burnetii* is very stable and easily transmitted in aerosols. Estimates of infectivity for humans range from 1 to 10 microorganisms.[7] Survival of the microorganisms for long periods in contaminated foods, buildings, and pastures has resulted in human and animal infections.

We became acutely aware of the resistance of *C. burnetii* to disinfectants during the production of a new-generation vaccine. Formalin treatment of purified *C. burnetii*, followed by chloroform-methanol extraction at 53°C, failed to destroy infectivity completely. As chemical disinfectants are not acceptable in vaccines, we resorted to gamma irradiation of vaccine materials and diagnostic reagents to ensure sterility.[8] These considerations stimulated us to reevaluate the effectiveness of some commonly used laboratory disinfectants.

Adequate disinfection of *C. burnetii* is commonly based on the assumption that disinfectants and procedures sufficient to inactivate *Bacillus* spores will also destroy *C. burnetii* infectivity. Our findings show that the susceptibility of *C. burnetii* to disinfectants does not equate with that of *Bacillus* spores. *C. burnetii* seeded onto membrane filters and exposed overnight to humidified formaldehyde gas or ethylene oxide within a small, sealed chamber were inactivated. However, formaldehyde gas failed to consistently inactivate more than $10^{4.6}$ *C. burnetii* in a large (5600 ft^3) room without humidity control; but 10^6 *Bacillus* spores similarly treated were inactivated. Liquid suspensions of 10^8 *C. burnetii* in 0.5% sodium hypochlorite, 2% Roccal, 5% Lysol, or 5% formalin were infectious after 24 h at 25°C. Similar suspensions in 70% ethyl alcohol, 5% chloroform, or 5% Enviro-Chem resulted in the inactivation of *C. burnetii* within 30 min.

MATERIALS AND METHODS

Test Cultures

Coxiella burnetii, Nine Mile strain in phase I, cloned in cell culture (9MIC7), were grown in yolk sacs of embryonated chicken eggs. Infected membranes were blended to a 50% (w/v) suspension in phosphate-buffered saline (PBS) and stored at −70°C. Microorganisms were separated from infected yolk sacs by Renografin density gradient centrifugation.[9]

Chemical Disinfectants

Lysol (*O*-phenylphenol, 2.80%; *O*-benzyl-*p*-chlorophenol, 2.70%; alcohol, 1.80%; xylenols, 1.5%; isopropyl alcohol, 0.90%; tetrasodium ethyl-diamine tetra-acetate, 0.76%; and soap, 16.5%), 11% sodium hypochlorite, formalin (40% formaldehyde), and chloroform were obtained from ordinary commercial sources. Alcide, an acidified sodium chlorite solution, was obtained from Alcide Corporation, Norwalk, CT; Enviro-Chem, a mixture of *N*-alkyl dimethyl benzyl (2.25%), and ethylbenzal (2.25%) ammonium chlorides was from Chem Sales, Ellicott City, MD; paraformaldehyde (flake) was from Celanese Chem Co., New York, NY; Oxyfume 12 (12% ethylene oxide, 88% dichlorodifluoromethane) was from Union Carbide Corp., Danbury, CT; Roccal (alkyl dimethyl benzyl ammonium chloride, 10%; ethyl alcohol, 1.25%) was from Sterling Drug Service, Montvale, NJ.

The purified *C. burnetii* was diluted to 10^8 infectious microorganisms per ml of disinfectant and held static at room temperature (24°C). After selected contact periods, the microorganisms were sedimented by centrifugation at 8000 × *g* for 30 min. The disinfectant solution was discarded, and the microorganisms were resuspended to their initial 20-ml volume in brain-heart–infusion (BHI) broth.

Gas Sterilization

The effectiveness of formaldehyde gas inactivation of *C. burnetii* and *B. subtilis* spores was tested by depolymerizing crystalline paraformaldehyde in a fry pan at 425°F. The microorganisms were diluted in BHI, seeded onto membrane filters, and permitted to dry in a class II biological safety cabinet (BSC) at concentrations ranging from $10^{4.6}$ to $10^{9.6}$ infectious *C. burnetii* per filter. Replicate filters with each concentration were exposed within a 5600-ft^3 laboratory room, a sealed 45-ft^3 class II BSC, and a sealed 88-ft^3 chamber. Filter paper patches seeded with 10^6 *B. subtilis* var. *niger* spores were simultaneously exposed within each facility. Samples were exposed to the formaldehyde gas overnight at room temperature, then removed and blended in BHI for sterility assays.

Filters seeded with *C. burnetii* were exposed to ethylene oxide gas in a modified 4-ft^3 autoclave. Water-saturated paper toweling, approximately 10 × 14 in, was placed in the autoclave chamber to provide moisture. The chamber was evacuated to −20 in Hg, then pressurized to +5 in Hg with Oxyfume 12. After overnight exposure (17 h) at room temperature (24°C), the samples were prepared for sterility assays.

Infectivity Assays

Treated samples were subpassaged in chick embryo yolk sacs and in mice. Ten 5-day-old chick embryos were each injected with 0.3 ml of sample. After incubation at 36°C for 8 d, the yolk-sacs were harvested and blended to a 50% (w/v) suspension in BHI; 0.5 ml was injected into each of five mice. After 4 weeks, individual mice were tested for seroconversion by enzyme-linked immunosorbent assay (ELISA).[10]

Two serial passages of treated *C. burnetii* were also carried out in mice. For each sample five mice were injected intraperitoneally with 0.5 ml of the sample. Eight days later, each mouse spleen was triturated in 5 ml of BHI, the five suspensions were pooled, and another five mice were injected with the pooled material. The five mice injected with second-passage mouse spleens were tested for seroconversion after 4 weeks. Detection of anti–*C. burnetii* antibody in serum from any mouse indicated that at least some infectious *C. burnetii* survived the disinfection procedure.

RESULTS AND DISCUSSION

The suspension of *C. burnetii* in aqueous solutions of Alcide, 0.5% hypochlorite, 5% Lysol, 5% formalin, or 2% Roccal did not completely inactivate infectious *C. burnetii* after 24 h at 24°C (TABLE 1). The number of surviving microorganisms in each sample was not quantitated, as we were only interested in assaying for the complete inactivation of *C. burnetii* by the respective disinfectants. The assays were designed to detect small numbers of infective *C. burnetii*. Results indicated that at least a few infectious *C. burnetii* escaped destruction in each solution, even though the tested disinfectant concentrations exceeded those recommended for general laboratory disinfection. By contrast, similar concentrations of *C. burnetii* were completely inactivated within 30 min by either 70% ethyl alcohol, 5% chloroform, or 5% Enviro-Chem.

Overnight exposure to humidified (80% relative humidity) formaldehyde gas, generated by depolymerizing paraformaldehyde at a rate of 0.3 g/ft^3 of space within a sealed 88-ft^3 chamber, effectively inactivated 10^8 *C. burnetii* on the surface of membrane filters (TABLE 2). However, in a large (5600 ft^3) laboratory room without positive humidity control, filters with more than $10^{4.6}$ *C. burnetii* were not consistently sterilized (TABLE 3). Depolymerization of twice the amount of paraformaldehyde (0.6 g/ft^3) within a closed, 45-ft^3 class II BSC at unknown relative humidity failed to consistently sterilize samples seeded with $>10^{7.6}$ *C. burnetii*. Overnight exposure to humidified 12% ethylene oxide gas at 24°C sterilized filters seeded with 10^8 *C. burnetii* (TABLE 2). Filters seeded with 10^6 *Bacillus* spores and subjected to the same test environments were all sterilized.

These data emphasize the resistance of *C. burnetii* to chemical disinfectants. The observed resistance in our test preparations of *C. burnetii*, which were completely free of host components, indicates that disinfection of samples containing organic matter would be formidable. Thus, contaminated birth tissues, fluids of parturient animal samples, and food products could neutralize the germicidal activity of efficacious disinfectants. Increased temperature and low pH are known to enhance the effectiveness of many disinfectants.[11] However, such conditions are not always acceptable alternatives for disinfecting research facilities or animal

TABLE 1. Inactivation of *C. burnetii* Suspended in Disinfectant Solutions

Disinfectant[a]	Infectivity after Contact Time[b] 0.5 h	24 h
Alcide (10 : 1 : 1)[c]	1/2	2/2
Sodium hypochlorite (0.5%)	2/2	2/2
Lysol (5%)	2/2	2/2
Roccal (2%)	2/2	2/2
Formalin (5%)	2/2	2/2
Ethyl alcohol (70%)	0/2	0/2
Chloroform (5%)	0/2	0/2
Enviro-Chem (5%)	0/2	0/2
None	2/2	2/2

[a] Active ingredients are listed in MATERIALS AND METHODS.
[b] Number of infectious samples/number tested. Test suspensions initially contained 10^8 infectious *C. burnetii* per ml of disinfectant solution.
[c] 10 parts water : 1 part base : 1 part activator, prepared as per manufacturer's instructions.

quarters contaminated with *C. burnetii*. The aqueous solutions of hypochlorite, formalin, Roccal, and Lysol tested did not inactivate high concentrations of *C. burnetii*, but perhaps more concentrated disinfectant solutions, or solutions containing alcohol, would be more effective. Ethyl alcohol (70%) was effective but, of course, evaporation would render it ineffectual.

The developmental cycle of *C. burnetii* produces large cells, with and without endogenous spore forms, and small dense cells. The proportion of the morphologically different forms carrying the endogenous spore is unknown. Each of these cell types may differ in its sensitivity to disinfectants. Samples containing high concentrations of organisms were more difficult to sterilize; it is likely that these samples contained larger numbers of the endogenous spore forms which are known to be difficult to inactivate.

TABLE 2. Inactivation of *C. burnetii* by Gaseous Formaldehyde and Ethylene Oxide

Treatment	Test Space (ft^3)	Amount of Disinfectant	Relative Humidity (%)	No. *C. burnetii*	Residual Infectivity[a]
Autoclaved[b]	NA[c]	NA[c]	NA[c]	10^5	0/2
				10^8	0/2
None	4.0	None	>50	10^5	2/2
				10^8	2/2
Paraformaldehyde[d]	88.0	26.4 g	80	10^5	0/4
				10^8	0/4
Ethylene oxide[d]	4.0	12% in Freon-12	>50	10^5	0/4
				10^8	0/4

[a] Number of infectious samples/total tests conducted.
[b] Membrane filters seeded with *C. burnetii* were autoclaved at 250°F and 21 psi for 15 min.
[c] NA, not applicable.
[d] After 17-h contact with the humidified gas at 24°C, filter samples were aseptically transferred to sterile BHI broth for viability assays.

Known germicidal mechanisms of chemical disinfectants for vegetative bacterial cells include the gross coagulation of cell proteins by strong disinfectants (strong solutions of formalin), less obvious denaturation of essential enzymes, rapid lysis of the bacterial cell, leakage of cell constituents without lysis (phenolic compounds), and more subtle mechanisms such as the oxidation of enzymes (iodine), and the gradual alteration of cell membranes by quaternary ammonium compounds.[12] A single disinfectant may exert several of these effects, depending on the concentration of the disinfectant and the type of microorganisms. Without doubt, several of the mechanisms are applicable to the large *C. burnetii* cells, but the "cidal" mechanisms effective for the endogenous spore form, or for the small dense cells of *C. burnetii*, are not known. Perhaps, as with *Bacillus* spores, cell-wall composition and thickness, and water content of the variant cell types are important.

Both formaldehyde and ethylene oxide gas effectively sterilized *C. burnetii* when high atmospheric moisture levels were maintained. The failure of formaldehyde gas to sterilize samples consistently in the larger room or biological safety

TABLE 3. Formaldehyde Gas Inactivation of *C. burnetii* on the Surface of Filters in a Large (Room) and Small (Class II BSC) Test Space

Infectious *C. burnetii* on Untreated Filters	Residual Infectivity[a]	
	Room (5600 ft³)	Class II BSC (45 ft³)
$10^{9.6}$	4/4	2/4
$10^{8.6}$	4/4	1/4
$10^{7.6}$	0/4	0/4
$10^{6.6}$	2/4	0/4
$10^{5.6}$	1/4	0/4
$10^{4.6}$	0/4	0/4

[a] Paraformaldehyde was depolymerized by heat at a rate of 0.3 g per ft³ of room or 0.6 g per ft³ of class II BSC space. Samples were exposed for 17 h at 24°C. The number of filters with infectious *C. burnetii* per number tested is shown.

cabinet emphasizes the need to establish appropriate moisture control. Although we raised the humidity by generating steam in the room, the humidity was not monitored or controlled in this experiment. Because formaldehyde does not inactivate *C. burnetii* consistently, we cannot recommend its use for sterilization. Although formaldehyde penetrates membranes, penetration of the large cells containing endogenous spores is probably not completely effective. Therefore, the few mature endogenous spores that escape cross-linking of their surface by formaldehyde are likely to cause infection in various hosts. The importance of hydrating contaminating microorganisms at the time of ethylene oxide disinfection has been demonstrated.[13] It was reported that non-uniform reactions of dried bacterial cells with ethylene oxide gas occurred, but uniformity of reactions could be restored by rehydrating the cells. Others[14] found that prehumidification of the area to be decontaminated was more effective in attaining sterility than was the simultaneous dissemination of water vapor and gas. Our findings and other available information emphasize the requirement to provide mechanisms to generate, monitor, and control the relative humidity in those areas to be subjected to gaseous disinfection.

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